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DNA barcoding of an endangered plant species, *salvadora oleoides*

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Salvadora oleoides Decne is an endangered plant species. This species is important as the plant is used for multiple purposes. Natives use the plants for their medicinal needs in treating rheumatic pains and coughs, and many others. This species is threatened by over-exploitation and deforestation or illegal logging, as well as its very slow growth rate, so the accurate identification of this species is important. Knowledge about threats or even distribution is limited, making identifying species more difficult. Therefore, this research aimed at DNA barcoding of endangered species *Salvadora oleoides* to assess their potential at the meaningful taxonomic level. This study aims to amplify the *rbc*L and *mat*K genes in *Salvadora oleoides* collected from the different regions of Pakistan. The plant DNA isolation was carried out by the CTAB method. PCR analysis was performed, which showed amplification of both *rbc*L and *mat*K regions. The amplified products were purified and sent for sequencing. The results revealed a 100% sequencing rate for *rbc*L; however, the *mat*K amplified product was not sequenced due to the high heterozygosity in this region, which might result in polymerase slippage during the Sanger sequencing method. The *rbc*L sequence was checked for homology by BLASTn and then submitted to NCBI for GenBank accession. The accession number assigned to *Salvadora oleoides* was OP046316 for *rbc*L.
Keywords: DNA Barcoding, rbcl, BLAST, Medicinal Plants, Species Identification, matk..

INTRODUCTION

An endangered species *Salvadora oleoides* from the Salvadoraceae family containing three genera has 12 species. This plant is used for multiple purposes, such as the oil extracted from the tree for medicinal purposes. It is distributed in Africa and subtropical and tropical areas of Asia mainly. The species is extremely valuable commercially; however, it is currently endangered due to overexploitation for wood and medicinal purposes. This plant has numerous alkaloids, glycosides, terpenoids, and flavonoids exploited for pharmacological activities (Khan *et al*., 2019).

The tree species are thought to be quickly diminishing due to a lack of suitable habitats, overexploitation, human activities, and agricultural land development (Khan, 1994). According to a 2012 study, *Salvadora oleoides*, which has a population of 11, is classified as Critically Endangered and in danger of going extinct (Khan *et al*., 2012). Tree species were evaluated for their conservation status based on their geographic distribution and the number of locations they were discovered in. *S. oleoides* was classified as being in the Endangered Category due to its restricted geographic range and solitary location. The geographic distribution of a tree species and the number of localities/subpopulations determine the species' conservation status. (Khan *et al*., 2012).

Plants of medicinal importance, such as *S. oleoides*, are typically sold in a dried form of leaves, dried roots, and tree barks at markets and traditional herbal shops or in processed powdered form in portions, mixes, or extracted material. Proper identification of the plants requires many morphological characteristics that are mostly missing for such plant material to identify by retailers and customers. Plant's Aerial parts might lose essential diagnostic features for taxonomical identification, while identification by root is usually difficult due to a lack of distinguishing morphology (Chen. *et al*., 2016). Furthermore, taxonomic identification utilizing macro- and micromorphological, as well as organoleptic approaches, can be time-taking, requires skill, and should be error-prone with accurate references (Li *et al*., 2011). Structural resemblances among some of the plant species and dried form of plant parts, the shortage of medicinally important plant species in wildlife, inconsiderate collection methods and practices, and the absence of a standard system of correct identification and control are major issues that contribute to both unintentional and deliberate replacement of species (Ghorbani *et al*., 2017).

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DNA barcoding proved to be an efficient tool for proper discrimination of the related species by generating standers that can be used universally. The discrimination is done by accurately sequencing the standard gene region in a very short time (Hebert *et al.*, 2003).

RbcL and matK have been having most recommended and vasty studied DNA barcodes for plants, as they are proved by PWG (Pair wise group) to be potential standardized barcodes for plant DNA barcoding. These barcodes are used to properly identify and discriminate any native or foreign plant species *(*Maloukh *et al*., 2017*)*. In some cases, *rbc*L showed less discriminatory power than matK but had other advantages of universal, clear alignment, unambiguity, and higher frequency for sequencing (Dong *et al*., 2014).

MATERIALS AND METHODS

Plant material: The plant species *Salvadora oleoides* was used in the study (Fig. 1). The germplasm of *Salvadora oleoides*, was obtained from different regions of Pakistan, including District of Mianwali, Bhakker Layyeh, MuzaffarGarh, some parts of the district of Khusab, Sargodha and Jhang. The plant material used during the experiment was a leaves sample *of Salvadora oleoides.*

Figure 1. Collected sample of *Salvadora oleoides.*

DNA extraction: DNA from young leaves of *Salvadora oleoides* was extracted using the modified CTAB method (Fig. 2). Samples of the plant's young leaves were taken and crushed using a sterile mortar and pestle. 1-2 ml of preheated CTAB Buffer was added and samples were grounded. To make the extracted DNA precipitate, chilled propanol was added. One µl β-Marcapto ethanol was added as the phenolic content was higher. The calculated volume of PCI and then phenol was used. After washing with 70% ethanol, Using the Nanodrop 8000, (Spectrophotometer, Thermo SCIENTIFIC) the quality of the isolated DNA was further evaluated at -20°C, the isolated DNA was kept safe..

Figure 2. Isolated DNA of *Salvadora oleoides* .

PCR analysis: The following ingredients were used in a 50 µL PCR reaction mixture: 3 µL of template DNA (300 ng), 5 µL of PCR buffer (10X), 5 µL of $MgCl_2$ (25 mM), 5µL of forward and reverse primers (10 pmol), 0.35 µL of Taq polymerase, 5 µL of dNTPs (1 mM), and the remaining was deionized distilled water. For PCR analysis, the heat profile and reagent profile were both standardized. In Salvadora oleoides, an endangered plant species in salty environments, the chloroplasts region rbcL and matK were employed as a DNA barcode for species identification.

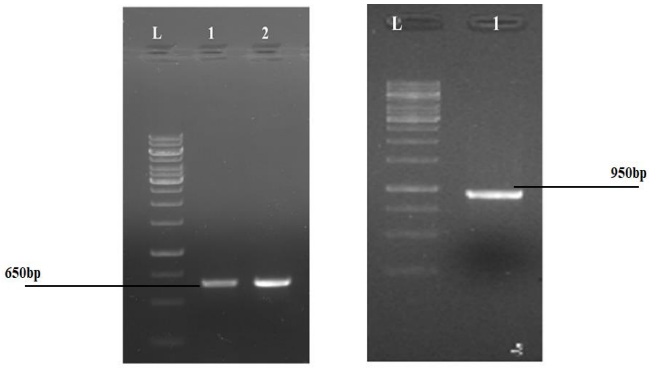
Nucleotide sequencing: The PCR-amplified products were extracted from the 1% TAE gel and purified with a DNA purification kit (Thermo Scientific). We tested the cleaned samples once again using a UV-2800 spectrophotometer (BMS).

The 20 L sequencing reaction was created using 2 L of template DNA (20 ng), 1 L of sequencing buffer (5X), and 2 L of a big dye terminator. 35 cycles of the thermal profile were added, with the temperatures being 95°C for 5 minutes, 94°C for 1 minute, 53°C for 45 seconds, 68°C for 4 minutes, and 68°C for 10 minutes for the final extension. 20 L was substituted for the final volume in place of sterile distilled water.

Two volumes of 80% propanol were used to precipitate the ordered products, and they were then given two washes with 80% ethanol. After air dried, the products were redissolved in 15 µL of formamide denaturing buffer. Sequencing the purified product, the nucleotide order was revealed, and these sequences were subsequently uploaded to the NCBI database. The GenBank database assigned the sequence an accession number. The biological sequence alignment editor BioEdit V 7.2.5 was used to trim the generated sequence.

RESULTS

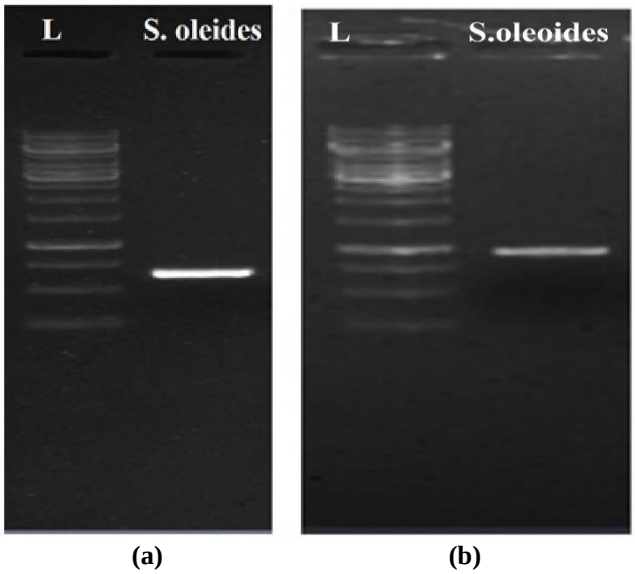
PCR analysis: PCR-based amplification of conserved regions is typically required for the generation of DNA barcodes for species identification (matK and rbcL). Both rbcL and matK are universal primers, and we saw successful PCR amplification of Salvadora oleoides using both of them. The amplification produced by both primers was effective.(Fig. 3).



(a) **(b)**

Figure 3. PCR analysis of *Salvadora oleoides* (a) *rbc*L (b) *mat*K *L=1kb Ladder .

Gel Purification: The amplified PCR product was purified from gel using the FavorPrep™ Gel purification mini kit (Fig. 4). Short primers, enzymes, dNTPs, PCR products, short-failed and salts are swiftly and efficiently removed from PCR fragments of greater than 100 bp using the PCR purification procedure, often in less than 10 minutes.After which, the purified product was checked on 1% agarose gel and, later on, sent for sequencing.



(a) **(b)**

Figure 4. Eluted products of *Salvadora oleoides* (a) *rbc*L, (b) *mat*K *L= 1kb Ladder .

Sequencing*:* Using the Basic Local Alignment Tool, the amplified sequences' sequence homology was found (BLAST). For matK, the amplified product couldn't get sequenced due to the high heterozygosity, which might have resulted in polymerase slippage during Sanger sequencing method. The sequence length of rbcL was 526 nucleotides.
The sequenced sections revealed conserved genomic data that will be used to identify various plants that belong to these species in the future. After sequencing analysis, the amplified conserved barcodes indicated various degrees of biological similarity.
Bioinformatics studies: Different Bioinformatic analyses were performed on the obtained sequence. The sequence was submitted to GenBank after homology confirmation through the BLASTn tool that showed homology with *Salvadora persica*. The BLAST (basic local alignment search tool) program is used in bioinformatics to compare primary biological sequence data, the nucleotides of DNA or RNA sequences. The accession number assigned to *Salvadora oleoides* was OP046316 for *rbc*L.
The results showed that accurate species identification and discrimination could be achieved using these conserved DNA sequences as barcode primers.

DISCUSSION

The genome sequence investigations of multiple species and the mapping of complex characteristics associated with diverse phenotypes allowed for the identification of several genes and their complex inheritance patterns in numerous plant species. DNA quality has a significant impact on PCR-based amplification. When metabolites are present in plants, they might occasionally affect the DNA quality during isolation, necessitating alternative DNA isolation techniques even for closely similar species (Khanuja *et al*., 1999). The fundamental method for determining a species' identity in plants is phylogenetic reconstruction and sequencing divergence from the reference sequence. Using DNA barcodes in native animals was a pioneering move toward developing DNA-based monitoring techniques for the adulteration of medicines in domestic and international commerce. However, many plant genomes are still missing sequencing information (Altschul *et al*., 1997). A genus-based identification strategy would be ideal when identifying native land plant species. Re-sequencing more loci for target-based upgrades may be helpful to uncover more genomic regions that are conserved across distinct plant species. The rbcL gene, which codes for a part of the crucial photosynthesis enzyme ribulose bisphosphate carboxylase, is found in almost all plant species.

Genes were amplified and sequenced in the Salvadora oleoides plant species' conserved areas for the current investigation. It is ideal for DNA barcoding since it contains a portion of its DNA sequence that differs significantly between species. The Group II intron splicing process requires the chloroplast's MaturaseK gene (MatK), which has shown extensive conservation in plant systematics (Notredame *et al.,* 2000). The gene is emerging as a viable option for the study of plant systematics and evolution since it has significant substitution rates within the species. So, this gene was also used for the DNA barcoding of *Salvadora oleoides.* The samples were collected from different regions of Pakistan. From the young leaves of *Salvadora oleoides*, DNA extraction was done by the modified CTAB method.

DNA barcoding of endangered plant species *S. oleoides* has been obtained in this study using the two most reliable and efficient DNA barcodes *rbc*L and *mat*K. The matK barcoding area presents a difficult amplification and sequencing problem because of the high sequence variation in the primer binding sites (Hollingsworth *et al.,* 2011). The extremely variable matK region has worse PCR amplification success than the more conserved rbcL gene, as seen by the numerous studies that have documented frequent PCR failures employing these primers (Kress and Erickson, 2007; Gonzalez *et al*., 2009). In this study, *mat*K showed 100% amplification result but the sample could not get sequenced. A similar result for the studies in which *mat*K cannot get sequenced were on *Durio graveolent* and *Durio zibetinus* (Cahyaningsih *et al*., 2021) and plants of the genus Acacia (Ismail *et al*., 2020).

In this research study, the species identification of *S. oleoides* has proven the strength of *rbc*L gene as the potential barcode. But, *mat*K showed less reproducibility. After the genes' sequences had been obtained, the signature DNA barcodes of this species were submitted to the NCBI. This study has proven the strength of *rbc*L as a suitable DNA barcode for the species identification of endangered species *Salvadora oleoides*.

***Conclusion*:** A unique *rbc*L sequence was found by amplifying and sequencing conserved genomic regions in engendered plant species of *Salvadora oleoides.* The study's conclusions might be used to develop DNA-based methods for classifying medicinal plant species and detecting adulteration.

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***Ethical statement*:** This article does not contain any studies with human participants or animal performed by any of the authors.

***Availability of data and material*:** We declare that the submitted manuscript is our work, which has not been published before and is not currently being considered for publication elsewhere.

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